REMARKS

Claims 29-34 were pending in the subject application. By this Amendment, applicant has amended claims 31, 32 and 34 so that they now depend from both claims 29 and 30, and added new claim 74. These amendments are supported in the specification at page 12, line 35 to page 13, line 7, as amended in the January 23, 2001 Preliminary Amendment, and thus raise no issue of new matter. Similarly, no new matter is introduced by the addition of new claim 74 which is fully supported in the specification at page 9, lines 17-19, as amended in the January 23, 2001 Preliminary Amendment. Accordingly, applicant respectfully requests that the Examiner enter this Amendment. Upon entry of this Amendment, claims 29-34 and 74, as amended, will be pending and under examination.

Applicant has amended the specification as indicated hereinabove to correct an obvious typographical error and thereby indicate that the B6B11 cell line does not secrete human IgG, IgM or IgA immunoglobulins. This correction is supported in the specification at, *inter alia*, page 30, lines 13-15; page 9, lines 15-19; page 10, lines 25-36; and page 54, lines 7-10. Applicant maintains, therefore, that correction of this obvious typographical error does not raise any issue of new matter.

The Invention

The invention claimed in the subject application provides methods of producing a monoclonal antibody from a tetroma cell formed by fusing a lymphoid cell capable of producing antibody with a trioma cell which does not produce any antibody, wherein the trioma cell is obtained by fusing a heteromyeloma cell which does not produce any antibody with a human lymphoid cell. The heteromyeloma cell used in this invention is obtained by fusing a human antibody-

nonproducing myeloma cell with a mouse antibody-nonproducing myeloma cell.

Rejections under 35 U.S.C. §112, First Paragraph

The Examiner rejected claims 29-33 under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement. The Examiner stated that the claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention.

The Examiner asserted that there is no support in the specification as originally filed for the recitation of "human antibody nonproducing myeloma cell" or "mouse antibody nonproducing myeloma cell" in claim 29 or 30. The Examiner stated that, regarding applicant's comments about page 29 of the specification, the cited passages of the specification are drawn to use of myeloma cell lines to produce B6B11 or B6B11-like cells (B6B11 cells were made from a fusion of HAT-sensitive, G-418 resistant myeloma X63.Ag8.653 myeloma cells and a subclone of human myeloma RPMI 8226 selected for nonsecretion of lambda light chains). The Examiner further stated that these passages do not disclose the production of other heteromyeloma cell lines and therefore the disclosure is not of the scope of the instant limitation which encompasses use of myeloma cell lines to produce heteromyeloma cells other than B6B11 or B6B11-like cells.

In addition, the Examiner stated that the cited passages do not disclose that the aforementioned cell lines are "antibody-nonproducing". The Examiner also stated that the disclosure indicates use of mouse myeloma cells without stating that the

mouse myeloma cells are antibody-nonproducing. The Examiner further stated that the specification does not indicate that the human myeloma cells used are "antibody-nonproducing", but discloses that they are selected for "non-secretion of antibody". The Examiner additionally stated that the quoted passage of the specification is silent as to whether the particular X63.Ag8.653 myeloma cell line used does or does not produce antibody.

In response, applicant respectfully traverses this rejection. Applicant notes that the claimed methods encompass the generation of a heteromyeloma cell which does not produce antibody, wherein the heteromyeloma cell is obtained by fusing a human antibody-nonproducing myeloma cell with a mouse antibody-nonproducing myeloma cell. These methods are fully supported in the specification at, *inter alia*, page 12, line 1 to page 13, line 7, as amended in the January 23, 2001 Preliminary Amendment.

The specification also describes on pages 29-30 the "specific generation and application of heteromyeloma B6B11" (see page 29, lines 9-10) as an example of a heteromyeloma cell which does not produce any antibody and which may be used in the claimed methods. See also the specification at page 9, lines 17-19, disclosing that the heteromyeloma B6B11 heteromyeloma cell is "an embodiment of [the] invention". Applicant asserts, however, that the claimed invention is not limited to the use of the B6B11 heteromyeloma or B6B11-like cells. As noted in the specification at page 28, lines 12-17, examples set forth in the Experimental Details section to illustrate the invention are not intended, and should not be construed, to limit in any way the invention as set forth in the claims.

Applicant asserts that the production of B6B11 and B6B11-like cells, as described on pages 29-30 of the specification, is

merely illustrative of the claimed methods for producing a heteromyeloma cell which does not produce any antibody, wherein the heteromyeloma cell is obtained by fusing a human antibody-nonproducing myeloma cell with a mouse antibody-nonproducing myeloma cell. Thus, the claimed methods are clearly not limited to the use of B6B11 and B6B11-like cells for the production of a trioma cell which is then used to produce a monoclonal antibody by fusing with an antibody-producing lymphoid cell. Applicant respectfully requests, therefore, that the Examiner reconsider and withdraw this ground of rejection.

Applicant also disagrees with the Examiner's statement that there is no support in the specification as originally filed for the recitation of "human antibody nonproducing myeloma cell" or "mouse antibody nonproducing myeloma cell" in claim 29 or 30. Applicant notes that the claimed methods of producing a monoclonal antibody employ a trioma cell which does not produce any antibody, wherein the trioma cell is obtained by fusing a heteromyeloma cell which does not produce any antibody with a human lymphoid cell. See the specification at, *inter alia*, page 12, lines 1-8; page 9, lines 15-17; and page 10, lines 13-23. The heteromyeloma cell which does not produce any antibody is in turn obtained by fusing a human antibody-nonproducing myeloma cell with a mouse antibody-nonproducing myeloma cell. In support, applicant respectfully directs the Examiner's attention to the specification at, *inter alia*, page 42, lines 6-12. This passage discloses that the human myeloma line, RPMI 8226, which produces human Ig lambda chains, was cultured in RPMI-C medium containing 10% FCS, and "a <u>non-producing</u> clone of [the] 8226 line was selected by cloning in ECM" (emphasis added). Moreover, the specification discloses at page 41, lines 8-11, that "production" of cytoplasmic light and/or heavy chains in hybridomas, B6B11 and the parental cell lines was estimated immunocytochemically using the peroxidase-anti-peroxidase system (PAP). Applicant

maintains, therefore, that the specification does disclose an antibody-nonproducing human myeloma cell line for generating the heteromyeloma.

Applicant acknowledges that the specification also teaches that one of the parental lines used to generate B6B11 was a subclone of human myeloma RPMI 8226 selected for nonsecretion of lambda light chains. See, e.g., page 29, lines 11-14 and 30-33. However, in the context of hybridoma technology, one skilled in the art would understand that an "antibody-nonproducing" cell line is synonymous with an "antibody-nonsecreting" cell line. Indeed, on page 22, lines 26-29, the specification indicates that the two terms are used synonymously. Accordingly, applicant maintains that there is no merit in the Examiner's position that the specification discloses that the human myeloma cells used are antibody-nonsecreting, but does not disclose that these cells are antibody-nonproducing.

Moreover, applicant notes that certain elements of the claims are well known in the art, and have been known for decades, dating back to the seminal publication of a method for making monoclonal antibodies by G. Köhler and C. Milstein (1975) Continuous cultures of fused cells secreting antibody of predefined specificity, Nature 256: 495-497 (copy attached hereto as **Exhibit A**). In that paper, the authors describe the fusion of "non-producing variants of myeloma lines" or "variants in which one of the parental chains is no longer expressed" to produce hybrid lines that produce different antibodies directed against the same antigen (see page 497, left column). Thus, for decades now, the production of monoclonal antibodies using cell-cell hybridization has been achieved by hybridizing an antibody-producing cell line with an antibody-nonproducing cell line. Applicant respectfully reminds the Examiner that "the 'written description' requirement must be applied in the context of the particular invention and

the state of the knowledge." *Capon v. Eshhar*, 418 F.3d 1349, 1358 (Fed. Cir. 2005). Thus, in cases where techniques are well known in the art and have become routine, the specification need not describe such techniques or explain the associated technical jargon in minute detail.

In response to the Examiner's statement that the specification is silent as to whether the particular X63.Ag8.653 myeloma cell line used does or does not produce antibody, applicant notes that the X63.Ag8.653 myeloma cell line was well known in the art, as of the March 19, 1998 priority date of the subject application, to be an antibody-nonproducing cell line. In this regard, applicant respectfully directs the Examiner's attention to the publication by J.F Kearney et al. (1979) "A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines." J. Immunol. 123: 1548-1550, the Abstract of which is attached hereto as **Exhibit B**. Kearney et al. (1979) describes the isolation of the X63.Ag8.653 cell line, and discloses that it is a subclone of the mouse myeloma cell line, P3-X63-Ag8, that does not express immunoglobulin heavy or light chains (see **Exhibit B**).

Applicant maintains that the specification need not disclose that X63.Ag8.653 is an antibody-nonproducing cell line since "a patent need not teach, and preferably omits, what is well known in the art." *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986). See also M.P.E.P. §2163.II.2.

The Examiner also stated that the specification discloses that the RPMI 8226 cell line does not secrete lambda light chains, not that the cell line does not secrete antibody. The Examiner asserted that this disclosure differs in scope from the limitation "antibody-nonproducing." The Examiner contended that

this is because "antibody-nonproducing" encompasses a variety of potential mechanisms whereby antibody is not produced (e.g., the cell line does not produce heavy chains, or lacks the ability to assemble and secrete antibody), and therefore the scope of the claim limitation is broader than the disclosure in the specification that the human myeloma does not secrete lambda light chains. The Examiner asserted that the instant disclosure also does not encompass human myeloma cells which do not produce antibody because they do not produce kappa light chains.

The Examiner concluded that there is no support in the specification as originally filed for the scope of the claimed invention, i.e., the claimed invention constitutes new matter.

In response, applicant notes that it is well known in the art that the only antibody-related polypeptides secreted by RPMI 8226 cells are free lambda light chains. See, e.g., Pickering and Gelder (1982) "A human myeloma cell line that does not express immunoglobulin but yields a high frequency of antibody-secreting hybridomas." J. Immunol. 129: 406-412, the Abstract of which is attached hereto as **Exhibit C**. Therefore, one skilled in the art would understand that a RPMI 8226 clone which does not secrete lambda light chains is a clone which does not produce any antibody.

Applicant directs the Examiner's attention to the publication by Rice and Baltimore (1982) Regulated expression of an immunoglobulin kappa gene introduced into a mouse lymphoid cell line, Proc. Natl. Acad. Sci. USA 79: 7862-7865 (copy attached hereto as **Exhibit D**), which describes introducing a recombinant light chain into a lymphoid cell that produces a heavy chain, and reconstituting a complete antibody. This demonstrates that in 1982, one skilled in the art understood the consequences of

expressing one of the heavy or light chains in a cell, versus expressing both chains.

Indeed, the Examiner's contention this "antibody-nonproducing" encompasses a variety of potential mechanisms underscores applicant's position that persons skilled in the art, including the Examiner, understand that a number of different conditions result in antibody nonproduction. In other words, the Examiner demonstrates the adequacy of the written description because, by relying on the general knowledge in the art, he is able to suggest that the specification should have set forth these different mechanisms. However, as noted above, since these mechanisms are all well known, it is not necessary to describe them in the specification. See *Hybritech, Inc. v. Monoclonal Antibodies, Inc., supra; Capon v. Eshhar, supra;* M.P.E.P. §2163.II.2. Thus, applicant maintains that the recitation of "antibody-nonproducing" cell lines in the claims does not constitute new matter.

Further, applicant reiterates that the specification fully supports the use of a parental cell line which does not produce (secrete) antibody to generate a heteromyeloma which does not produce (secrete) antibody. It is immaterial whether the mechanism by which the parental cell line fails to produce antibody is that, for example, it does not produce heavy chains, or does not produce kappa light chains, or lacks the ability to assemble and secrete antibody. Applicant also reiterates that the claimed invention is not restricted to methods employing B6B11 or B6B11-like cells, which cells are formed from a human myeloma (RPMI 8226) which does not secrete lambda light chains.

Applicant respectfully submits that the foregoing remarks obviate the rejection of claims 29 and 30 for lack of written description, and requests that the Examiner reconsider and

withdraw the stated grounds of rejection.

Applicant maintains that the remarks made above in relation to claims 29 and 30 also obviate the rejections of claims 31-33. Accordingly, applicant also requests that the Examiner reconsider and withdraw the grounds of rejection of claims 31-33.

Objection

The Examiner objected to claim 34 as dependent upon a rejected base claim, but stated that this claim would be allowable if rewritten in independent form.

In response, applicant respectfully submits that based on the remarks made herein, the rejection of base claim 29 should be withdrawn, thereby rendering the instant objection moot.

Conclusion

In view of the remarks set forth above, applicant respectfully requests that the Examiner reconsider and withdraw the grounds of rejection and objection set forth in the October 11, 2005 Office Action, and earnestly solicits allowance of the claims now pending in the subject application.

If a telephone conference would be of assistance in advancing the prosecution of the subject application, applicant's undersigned attorneys invite the Examiner to telephone them at the number provided below.

No fee is deemed necessary in connection with the filing of this Amendment. However, if any fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450 Alexandria, VA 22313-1450

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Continuous cultures of fused cells secreting antibody of predefined specificity

THE manufacture of predefined specific antibodies by means of permanent tissue culture cell lines is of general interest. There are at present a considerable number of permanent cultures of myeloma cells[1,2] and screening procedures have been used to reveal antibody activity in some of them. This, however, is not a satisfactory source of monoclonal antibodies of predefined specificity. We describe here the derivation of a number of tissue culture cell lines which secrete anti-sheep red blood cell (SRBC) antibodies. The cell lines are made by fusion of a mouse myeloma and mouse spleen cells from an immunised donor. To understand the expression and interactions of the Ig chains from the parental lines, fusion experiments between two known mouse myeloma lines were carried out.

Each immunoglobulin chain results from the integrated expression of one of several *V* and *C* genes coding respectively for its variable and constant sections. Each cell expresses only one of the two possible alleles (allelic exclusion; reviewed in ref. 3). When two antibody-producing cells are fused, the products of both parental lines are expressed[4,5], and although the light and heavy chains of both parental lines are randomly joined, no evidence of scrambling of *V* and *C* sections is observed[4]. These results, obtained in an heterologous system involving cells of rat and mouse origin, have now been confirmed by fusing two myeloma cells of the same mouse strain, and provide the background for the derivation and understanding of antibody-secreting hybrid lines in which one of the parental cells is an antibody-producing spleen cell.

Two myeloma cell lines of BALB/c origin were used. P1Bul is resistant to 5-bromo-2′-deoxyuridine[4], does not grow in selective medium (HAT, ref. 6) and secretes a myeloma protein, Adj PC5, which is an IgG2A (κ), (ref. 1). Synthesis is not balanced and free light chains are also secreted. The second cell line, P3-X63Ag8, prepared from P3 cells[8], is resistant to 20 μg ml^{-1} 8-azaguanine and does not grow in HAT medium. The protein secreted (MOPC 21) is an IgG1 (κ) which has been fully sequenced[7,8]. Equal numbers of cells from each parental line were fused using inactivated Sendai virus[9] and samples contining 2×10^5 cells were grown in selective medium in separate dishes. Four out of ten dishes showed growth in selective medium and these were taken as independent hybrid lines, probably derived from single fusion events. The karyotype of the hybrid cells after 5 months in culture was just under the sum of the two parental lines (Table 1). Figure 1 shows the isoelectric focusing[10] (IEF) pattern of the secreted products of different lines. The hybrid cells (samples *c–h* in Fig. 1) give a much more complex pattern than either parent (*a* and *b*) or a mixture of the parental lines (*m*). The important feature of the new pattern is the presence of extra bands (Fig. 1, arrows). These new bands, however, do not seem to be the result of differences in primary structure; this is indicated by the IEF pattern of the products after reduction to separate the heavy and light chains (Fig. 1*B*). The IEF pattern of chains of the hybrid clones (Fig. 1*B*, *g*) is equivalent to the sum of the IEF pattern (*a* and *b*) of chains of the parental clones with no evidence of extra products. We conclude that, as previously shown with interspecies hybrids[4,5], new Ig molecules are produced as a result of mixed association between heavy and light chains from the two parents. This process is intracellular as a mixed cell population does not give rise to such hybrid molecules (compare *m* and *g*, Fig. 1*A*). The individual cells must therefore be able to express both isotypes. This result shows that in hybrid cells the expression of one isotype and idiotype does not exclude the expression of another: both heavy chain isotypes (γ1 and γ2a) and both V_H and both V_L regions (idiotypes) are expressed. There are no allotypic markers for the C_K region to provide direct proof for the expression of both parental C_K regions. But this is indicated by the phenotypic link between the *V* and *C* regions.

Figure 1*A* shows that clones derived from different hybridisation experiments and from subclones of one line are indistinguishable. This has also been observed in other experiments (data not shown). Variants were, however, found in a survey of 100 subclones. The difference is often associated with changes

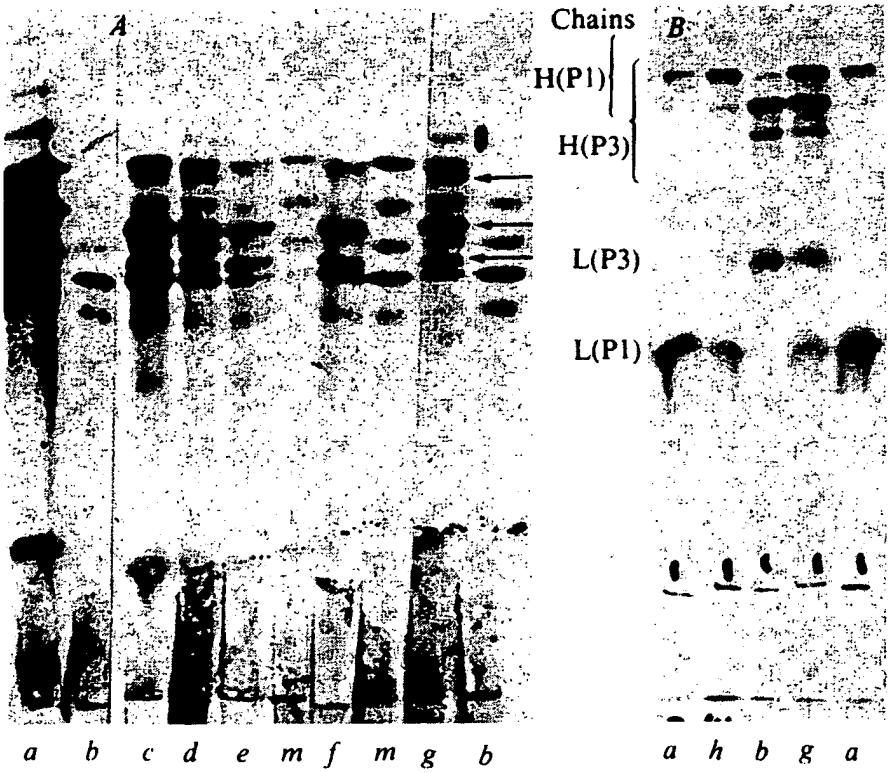

Fig. 1 Autoradiograph of labelled components secreted by the parental and hybrid cell lines analysed by IEF before (*A*) and after reduction (*B*). Cells were incubated in the presence of ^{14}C-lysine[11] and the supernatant applied on polyacrylamide slabs. *A*, *p*H range 6.0 (bottom) to 8.0 (top) in 4 M urea. *B*, *p*H range 5.0 (bottom) to 9.0 (top) in 6 M urea; the supernatant was incubated for 20 min at 37 °C in the presence of 8 M urea, 1.5 M mercaptoethanol and 0.1 M potassium phosphate *p*H 8.0 before being applied to the right slab. Supernatants from parental cell lines in: *a*, P1Bul; *b*, P3-X67Ag8; and *m*, mixture of equal number of P1Bul and P3-X67Ag8 cells. Supernatants from two independently derived hybrid lines are shown: *c–f*, four subclones from Hy-3; *g* and *h*, two subclones from Hy-B. Fusion was carried out[4,9] using 10^6 cells of each parental line and 4,000 haemagglutination units inactivated Sendai virus (Searle). Cells were divided into ten equal samples and grown separately in selective medium (HAT medium, ref. 6). Medium was changed every 3 d. Successful hybrid lines were obtained in four of the cultures, and all gave similar IEF patterns. Hy-B and Hy-3 were further cloned in soft agar[14]. L, Light; H, heavy.

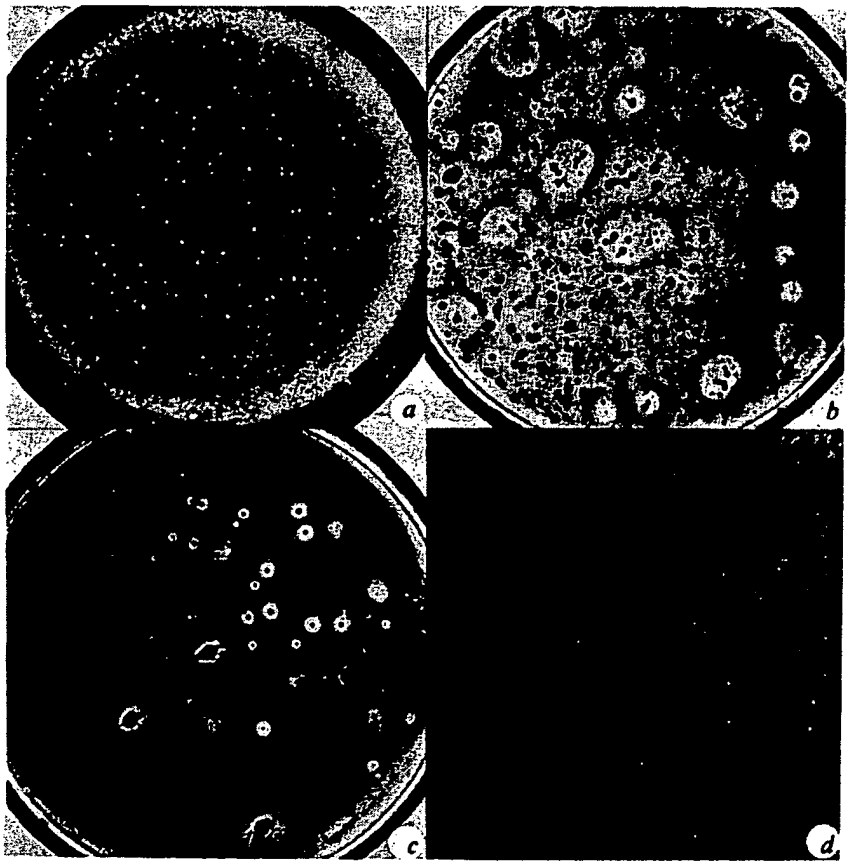

Fig. 2 Isolation of an anti-SRBC antibody-secreting cell clone. Activity was revealed by a halo of haemolysed SRBC. Direct plaques given by: *a*, 6,000 hybrid cells Sp-1; *b*, clones grown in soft agar from an inoculum of 2,000 Sp-1 cells; *c*, recloning of one of the positive clones Sp-1/7; *d*, higher magnification of a positive clone. Myeloma cells (10^7 P3-X67A g8) were fused to 10^8 spleen cells from an immunised BALB/c mouse. Mice were immunised by intraperitoneal injection of 0.2 ml packed SRBC diluted 1:10, boosted after 1 month and the spleens collected 4 d later. After fusion, cells (Sp-1) were grown for 8 d in HAT medium, changed at 1–3 d intervals. Cells were then grown in Dulbecco modified Eagle's medium, supplemented for 2 weeks with hypoxanthine and thymidine. Forty days after fusion the presence of anti-SRBC activity was revealed as shown in *a*. The ratio of plaque forming cells/total number of hybrid cells was 1/30. This hybrid cell population was cloned in soft agar (50% cloning efficiency). A modified plaque assay was used to reveal positive clones shown in *b–d* as follows. When cell clones had reached a suitable size, they were overlaid in sterile conditions with 2 ml 0.6% agarose in phosphate-buffered saline containing 25 µl packed SRBC and 0.2 ml fresh guinea pig serum (absorbed with SRBC) as source of complement. *b*, Taken after overnight incubation at 37 °C. The ratio of positive/total number of clones was 1/33. A suitable positive clone was picked out and grown in suspension. This clone was called Sp-1/7, and was recloned as shown in *c*; over 90% of the clones gave positive lysis. A second experiment in which 10^8 P3-X67Ag8 cells were fused with 10^8 spleen cells was the source of a clone giving rise to indirect plaques (clone Sp-2/3-3). Indirect plaques were produced by the addition of 1:20 sheep anti-MOPC 21 antibody to the agarose overlay.

in the ratios of the different chains and occasionally with the total disappearance of one or other of the chains. Such events are best visualised on IEF analysis of the separated chains (for example, Fig. 1*h*, in which the heavy chain of P3 is no longer observed). The important point that no new chains are detected by IEF complements a previous study[4] of a rat–mouse hybrid line in which scrambling of *V* and *C* regions from the light chains of rat and mouse was not observed. In this study, both light chains have identical C_κ regions and therefore scrambled V_L–C_L molecules would be undetected. On the other hand, the heavy chains are of different subclasses and we expect scrambled V_H–C_H to be detectable by IEF. They were not observed in the clones studied and if they occur must do so at a lower frequency. We conclude that in syngeneic cell hybrids (as well as in interspecies cell hybrids) *V*–*C* integration is not the result of cytoplasmic events. Integration as a result of DNA translocation or rearrangement during transcription is also suggested by the presence of integrated mRNA molecules[11] and by the existence of defective heavy chains in which a deletion of *V* and *C* sections seems to take place in already committed cells[12].

The cell line P3-X63Ag8 described above dies when exposed to HAT medium. Spleen cells from an immunised mouse also die in growth medium. When both cells are fused by Sendai virus and the resulting mixture is grown in HAT medium, surviving clones can be observed to grow and become established after a few weeks. We have used SRBC as immunogen, which enabled us, after culturing the fused lines, to determine the presence of specific antibody-producing cells by a plaque assay technique[13] (Fig. 2*a*). The hybrid cells were cloned in soft agar[14] and clones producing antibody were easily detected by an overlay of SRBC and complement (Fig. 2*b*). Individual clones were isolated and shown to retain their phenotype as almost all the clones of the derived purified line are capable of lysing SRBC (Fig. 2*c*). The clones were visible to the naked eye (for example, Fig. 2*d*). Both direct and indirect plaque assays[13] have been used to detect specific clones and representative clones of both types have been characterised and studied.

The derived lines (Sp hybrids) are hybrid cell lines for the following reasons. They grow in selective medium. Their karyotype after 4 months in culture (Table 1) is a little smaller than the sum of the two parental lines but more than twice the chromosome number of normal BALB/c cells, indicating that the lines are not the result of fusion between spleen cells. In addition the lines contain a metacentric chromosome also present in the parental P3-X67Ag8. Finally, the secreted immunoglobulins contain MOPC 21 protein in addition to new, unknown components. The latter presumably represent the chains derived from the specific anti-SRBC antibody. Figure 3*A* shows the IEF pattern of the material secreted by two such Sp hybrid clones. The IEF bands derived from the parental P3 line are visible in the pattern of the hybrid cells, although obscured by the presence of a number of new bands. The pattern is very complex, but the complexity of hybrids of this type is likely to result from the random recombination of chains (see above, Fig. 1). Indeed, IEF patterns of the reduced material secreted by the spleen–P3 hybrid clones gave a simpler pattern of Ig chains. The heavy and light chains of the P3 parental line became prominent, and new bands were apparent.

The hybrid Sp-1 gave direct plaques and this suggested that it produces an IgM antibody. This is confirmed in Fig. 4 which shows the inhibition of SRBC lysis by a specific anti-IgM

Table 1 Number of chromosomes in parental and hybrid cell lines

Cell line	Number of chromosomes per cell	Mean
P3-X67Ag8	66,65,65,65,65	65
PIBul	Ref. 4	55
Mouse spleen cells	—	40
Hy-B (P1–P3)	112,110,104,104,102	106
Sp-1/7-2	93,90,89,89,87	90
Sp-2/3-3	97,98,96,96,94,88	95

antibody. IEF techniques usually do not reveal 19S IgM molecules. IgM is therefore unlikely to be present in the unreduced sample *a* (Fig. 3*B*) but μ chains should contribute to the pattern obtained after reduction (sample *a*, Fig. 3*A*).

The above results show that cell fusion techniques are a powerful tool to produce specific antibody directed against a predetermined antigen. It further shows that it is possible to isolate hybrid lines producing different antibodies directed against the same antigen and carrying different effector functions (direct and indirect plaque).

The uncloned population of P3–spleen hybrid cells seems quite heterogeneous. Using suitable detection procedures it should be possible to isolate tissue culture cell lines making different classes of antibody. To facilitate our studies we have used a myeloma parental line which itself produced an Ig. Variants in which one of the parental chains is no longer expressed seem fairly common in the case of P1–P3 hybrids (Fig. 1*h*). Therefore selection of lines in which only the specific antibody chains are expressed seems reasonably simple. Alternatively, non-producing variants of myeloma lines could be used for fusion.

We used SRBC as antigen. Three different fusion experiments were successful in producing a large number of antibody-producing cells. Three weeks after the initial fusion, 33/1,086 clones (3%) were positive by the direct plaque assay. The cloning efficiency in the experiment was 50%. In another experiment, however, the proportion of positive clones was considerably lower (about 0.2%). In a third experiment the hybrid population was studied by limiting dilution analysis. From 157 independent hybrids, as many as 15 had anti-SRBC activity. The proportion of positive over negative clones is remarkably high. It is possible that spleen cells which have been triggered during immunisation are particularly successful in giving rise to viable hybrids. It remains to be seen whether similar results can be obtained using other antigenes.

The cells used in this study are all of BALB/c origin and the hybrid clones can be injected into BALB/c mice to produce solid tumours and serum having anti-SRBC activity. It is possible to hybridise antibody-producing cells from different origins[4,5]. Such cells can be grown *in vitro* in massive cultures to provide specific antibody. Such cultures could be valuable for medical and industrial use.

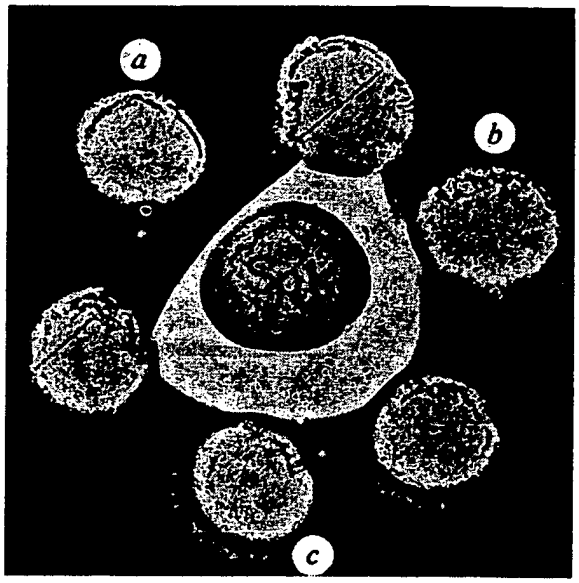

Fig. 4 Inhibition of haemolysis by antibody secreted by hybrid clone Sp-1/7-2. The reaction was in a 9-cm Petri dish with a layer of 5 ml 0.6% agarose in phosphate-buffered saline containing 1/80 (v/v) SRBC. Centre well contains 2.5 μl 20 times concentrated culture medium of clone Sp-1/7-2 and 2.5 μl mouse serum. *a*, Sheep specific anti-mouse macroglobulin (MOPC 104E, Dr Feinstein); *b*, sheep anti-MOPC 21 (P3) IgG1 absorbed with Adj PC-5; *c*, sheep anti-Adj PC-5 (IgG2a) absorbed with MOPC 21. After overnight incubation at room temperature the plate was developed with guinea pig serum diluted 1:10 in Dulbecco's medium without serum.

Fig. 3 Autoradiograph of labelled components secreted by anti-SRBC specific hybrid lines. Fractionation before (*B*) and after (*A*) reduction was by IEF. *p*H gradient was 5.0 (bottom) to 9.0 (top) in the presence of 6 M urea. Other conditions as in Fig. 1. Supernatants from: *a*, hybrid clone Sp-1/7-2; *b*, hybrid clone Sp-2/3-3; *c*, myeloma line P3-X67Ag8.

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ARTICLES

A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines

JF Kearney, A Radbruch, B Liesegang and K Rajewsky

We have isolated a subclone of the mouse myeloma cell line P3-X63-Ag8 that does not express immunoglobulin heavy or light chains. This clone X63-Ag8.653 can be used for efficient fusion with antibody-forming cells to obtain hybrid cell lines producing pure monoclonal antibodies. Screening of hybrid cell lines for specificity and immunoglobulin classes was done with a modified enzyme-linked immunosorbent assay.

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ARTICLES

A human myeloma cell line that does not express immunoglobulin but yields a high frequency of antibody-secreting hybridomas

JW Pickering and FB Gelder

We selected an 8-azaguanine-resistant variant of a human myeloma cell line (RPMI 8226) by cloning the parental cells on a feeder layer of mouse spleen cells in the presence of increasing concentrations of 8- azaguanine. Culture media and cellfree extracts of both the parental and variant (8226 AR/NIP4-1) cell lines were assayed for production of immunoglobulin heavy and light chains by double immunodiffusion and for lambda-chain by radioimmunoassay. Secretion of free lambda-chain by the parental cell line was confirmed. In contrast, no immunoglobulin heavy or light chains were detected in culture medium of the variant cell line by either immunodiffusion or radioimmunoassay. No intracellular lambda-chain could be detected in the variant cells by radioimmunoassay of cellfree extracts or by immunofluorescence of fixed cells. Hybridomas were produced by fusion of 8226AR/NIP4-1 cells with lymphocytes from a mesenteric lymph node recovered at surgery from a hypertransfused renal transplant recipient. Twenty hybrid culture supernatants were assayed for immunoglobulin by double immunodiffusion, and 15 contained either IgG (lambda) or IgG (kappa). None produced IgM or IgA. An IgG (kappa)-producing hybridoma was shown by immunofluorescence not to express lambda-chain. A second fusion between the variant cell line and spleen cells from a renal transplant patient produced a stable hybridoma secreting IgM (lambda) antibody specific for the I antigen.

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Regulated expression of an immunoglobulin κ gene introduced into a mouse lymphoid cell line

(Abelson murine leukemia virus/DNA transfection/*gpt* selection/lipopolysaccharide induction)

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Contributed by David Baltimore, September 16, 1982

ABSTRACT **We have introduced a functionally rearranged murine κ light chain immunoglobulin (Ig) gene into an Abelson murine leukemia virus-transformed lymphoid cell line. Plasmid pSV2gpt-κ41, containing the κ light chain gene from the myeloma MOPC41 and the selectable marker gene *gpt*, was introduced into 81A-2 cells by the calcium phosphate coprecipitation technique. Cells expressing the *gpt* gene were selected by growth in medium containing mycophenolic acid. One transfected cell line, κ-2, was shown to make κ mRNA and polypeptide chains and to assemble the κ chain product with γ2b heavy chains to form an apparently complete IgG2b. When bacterial lipopolysaccharide was added to the growth medium, levels of κ mRNA and polypeptide increased, showing regulated expression of the introduced κ gene.**

B cell differentiation proceeds from the "pre-B" lymphocyte, which synthesizes μ immunoglobulin (Ig) heavy chains but no light chains, to the mature B lymphocyte, which synthesizes both heavy and light chains and expresses surface Ig, and finally to the Ig-secreting plasma cell (1–5). The availability of transformed cell analogs has allowed biochemical characterization of these stages of cellular differentiation (6–11). Recently, such studies have contributed greatly to our understanding of the structure of Ig gene segments and the joining of these segments to produce a functionally rearranged Ig gene (12–17).

Although much is now known about Ig gene structure, relatively little is known about the molecular mechanisms that control Ig gene expression. One approach to study such controls is to introduce Ig genes into various cell lines, including both lymphoid cells at various stages of differentiation and nonlymphoid cells. One might then be able to identify control mechanisms unique to lymphoid cells that allow the cells to express, assemble, and secrete Igs. To begin such studies, we have attempted to introduce a functionally rearranged murine κ light chain gene into an Abelson murine leukemia virus (A-MuLV)-transformed lymphoid cell line.

Previous studies have shown that A-MuLV infection of bone marrow or fetal liver cells transforms cells of the B-lymphoid lineage, usually "pre-B" cells (18, 19). Derivatives of one A-MuLV transformant, 18-8, have been shown to switch from μ to γ2b heavy chain synthesis while in culture (20–22). One such derivative, 81A-2, synthesizes γ2b protein, but has lost its κ constant region light chain gene segments (unpublished data). Here we report that, after the introduction of a functionally rearranged κ gene into 81A-2 cells, the κ gene is expressed in a regulated manner.

MATERIALS AND METHODS

Cells. The A-MuLV-transformed cell line 81A-2, a derivative of the line 18-8, synthesizes γ2b heavy chain protein, but no light chain, and has lost its κ constant region genes (refs. 18 and 22; unpublished data). Cells were grown and analyzed for Ig protein synthesis by metabolic labeling and immunoprecipitation as described (18). Nonreduced samples were prepared for electrophoresis as described by Margulies *et al.* (23).

DNA Procedures. The phage λCh4A-41KC21, containing the rearranged genomic κ light chain gene from the myeloma MOPC41, was obtained from P. Leder (12). The 7-kilobase-pair (kbp) *Eco*RI/*Bam*HI fragment containing the κ gene was inserted into *Eco*RI- and *Bam*HI-cleaved plasmid pSV2gpt, obtained from R. Mulligan (24). The resulting plasmid, shown in Fig. 1, is called pSV2gpt-κ41. Ten micrograms of DNA from this plasmid was transfected into 5×10^7 81A-2 cells by a modification of the calcium phosphate technique of Graham and Van der Eb (25). Cells were washed in phosphate-buffered saline (0.14 M NaCl/2.5 mM KCl/16 mM Na_2HPO_4/1.4 mM KH_2PO_4), resuspended in 1 ml of transfection cocktail [made by adding DNA to 1 ml of Hepes-buffered saline, then adding 62.5 μl of 2 M $CaCl_2$ (26)] and incubated 15 min at room temperature. Then 10 ml of medium was added and the cells were incubated at 37°C for 4 hr. Cells were then washed in phosphate-buffered saline, incubated at 37°C for 2 min in 2 ml of Hepes-buffered saline with glycerol (26), and washed again in phosphate-buffered saline. Cells were then resuspended in 10 ml of nonselective medium, grown for 3 days, and then transferred to selective medium [RPMI 1640 medium supplemented with mycophenolic acid at 2 μg/ml, xanthine at 250 μg/ml, hypoxanthine at 15 μg/ml, and glutamine at 150 μg/ml (27)]. Transfected and mock-transfected 81A-2 cells were passaged in selective medium for approximately 3 weeks, until the mock-transfected cells had died. The transfected cells were then cloned by limiting dilution in nonselective medium.

RNA. Total cellular poly(A)-containing RNA was isolated by the guanidine·HCl procedure (28), fractionated according to size by electrophoresis in formaldehyde gels (29), transferred to nitrocellulose, and hybridized with ^{32}P-labeled DNA probes as described (30).

RESULTS

To examine expression of a κ gene from transfected plasmid DNA, the plasmid pSV2gpt-κ41 was constructed to contain the rearranged chromosomal κ light chain gene from the myeloma MOPC41 (12) and the selectable marker gene *gpt*, the gene from *Escherichia coli* that codes for the enzyme xanthine-guanine phosphoribosyltransferase [GPT; EC 2.4.2.22 (27)] (Fig. 1). In mammalian cells grown in media containing inhibitors of purine synthesis (here, mycophenolic acid), expression of the *gpt* gene allows selective cell growth using xanthine as the pre-

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Abbreviations: A-MuLV, Abelson murine leukemia virus; GPT, xanthine-guanine phosphoribosyltransferase; kb, kilobase(s); kbp, kilobase pair(s); LPS, bacterial lipopolysaccharide; SV40, simian virus 40.

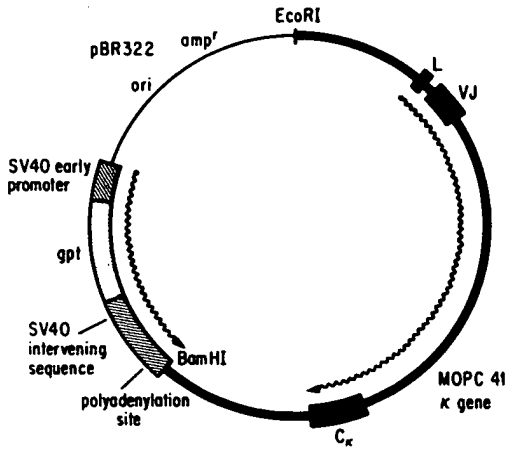

FIG. 1. Structure of the plasmid pSV2gpt-κ41 (11.4 kbp). Mouse DNA sequences containing the rearranged κ light chain gene from the myeloma MOPC41 are represented by the heavy dark line. The leader (L), variable plus joining region (VJ), and constant region (C_κ) of the κ gene are indicated. Simian virus 40 (SV40) sequences, represented by hatched regions, include DNA segments containing the early promoter, the small tumor antigen intervening sequence, and sequences for termination and polyadenylylation of SV40 early transcripts. The *gpt* gene from *Escherichia coli* is shown as a stippled region. *ori*, Origin of replication; *amp*^{r}, ampicillin resistance. Transcription units are indicated by wavy lines.

cursor for synthesis of guanine nucleotides (27). In pSV2gpt, the parental plasmid used for this construction, the *gpt* gene is transcribed from the SV40 early promoter and is followed by a region of SV40 DNA containing the small tumor antigen intervening sequence and signal sequences for transcript termination and polyadenylylation (24). To reduce the possibility of transcription of the κ light chain gene from promoters other than its own, plasmid pSV2gpt-κ41 was designed so that transcription from the SV40 promoter is in opposite orientation from that required for κ gene expression.

The 81A-2 cell line used as recipient of the transfected DNA is an A-MuLV-transformed murine lymphoid cell that synthesizes γ2b heavy chain but no light chain [no C_κ alleles can be detected by hybridization (ref. 22 and unpublished data)]. Plasmid pSV2gpt-κ41 DNA was introduced into 81A-2 cells by the calcium phosphate coprecipitation technique (25). Cells expressing the *gpt* gene were selected by growth in medium containing mycophenolic acid and then cloned by limiting dilution. When DNA from three selected cell lines was prepared and analyzed by hybridization with a κ probe, all three lines were found to have acquired one or a small number of the introduced κ genes. From the pattern of the hybridizing bands, at least two of three lines were judged to be independent transfectants. Eight cell lines were assayed for GPT enzyme activity by the *in situ* gel assay of Mulligan and Berg (24); all eight were positive (data not shown).

When the eight *gpt*$^+$ cell lines were assayed for production of κ protein by metabolic labeling with [^{35}S]methionine and immunoprecipitation with anti-κ antiserum, all eight were found to synthesize a polypeptide which (*i*) was precipitable with anti-κ antiserum (Fig. 2, lane d for clone κ-2 and data not shown for the others); (*ii*) comigrated with the κ chain produced by the myeloma MPC11 (apparent M_r 23,000) (Fig. 2, lane a); and (*iii*) was not evident in the nontransfected 81A-2 parent cell line (Fig. 2, lane b). In the original autoradiogram, the background bands in the M_r 23,000 region are much fainter than reproduced here. Because the 81A-2 cells lack C_κ alleles, none of the background bands are κ light chain. Precipitation of the M_r 23,000

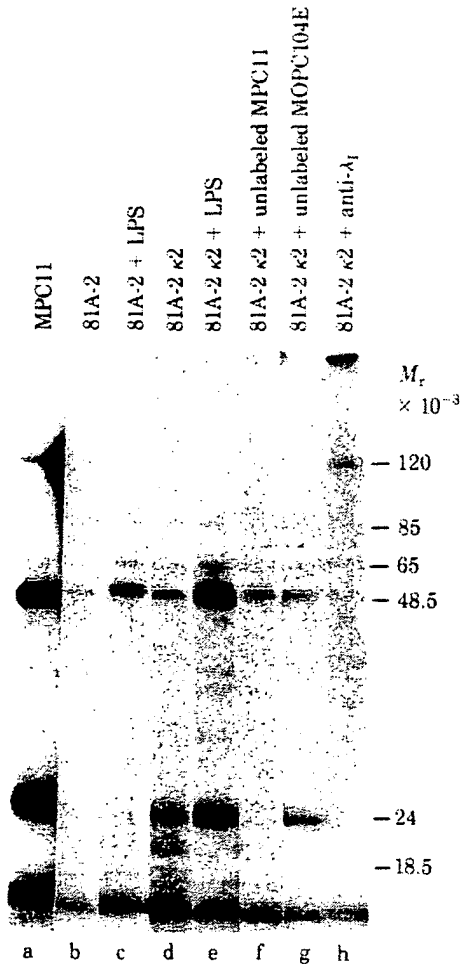

FIG. 2. Polyacrylamide gel electrophoresis of Ig polypeptides synthesized by 81A-2 cells and the transfectant κ-2. Cytoplasmic extracts were prepared from cells labeled with [^{35}S]methionine, immunoprecipitated, and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Lane a, myeloma MPC11 extract immunoprecipitated with anti-κ antiserum; lanes b and c, extract from parent A-MuLV-transformant 81A-2 grown in the absence (lane b) and presence (lane c) of *Salmonella typhimurium* lipopolysaccharide (LPS) and immunoprecipitated with anti-κ antiserum; lanes d and e, extract from the transfectant κ-2 grown in the absence (lane d) and presence (lane e) of LPS and immunoprecipitated with anti-κ antiserum; lanes f and g, extract from the transfectant κ-2 mixed with unlabeled MPC11 extract (lane f) or unlabeled MOPC104E extract (lane g) and immunoprecipitated with anti-κ antiserum; lane h, extract from the transfectant κ-2 immunoprecipitated with anti-λ_I antiserum. Sizes of molecular weight marker proteins are indicated.

polypeptide by anti-κ serum was blocked by competition with an unlabeled MPC11 protein extract (containing authentic κ light chains) but not by an unlabeled MOPC104E protein extract (containing λ_I light chains) (Fig. 2, lanes f and g). Also, the apparent κ chain was not precipitable by anti-λ_I antiserum (Fig. 2, lane h). Therefore, the M_r 23,000 polypeptide appears to be the protein product of the κ light chain gene transfected into the 81A-2 cells.

To examine the RNA produced from the transfected κ gene, poly(A)-containing mRNA was prepared from the parent 81A-2 cell line and the transfectant κ-2. The RNA was size-fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled plasmid DNA probe containing the κ constant region (C_κ), the κ joining (J_κ) segments, and the sequence that intervenes between them. No hybridization was detected to the RNA prepared from the par-

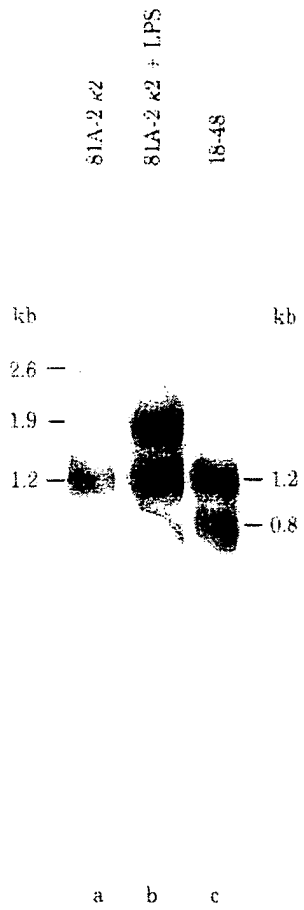

FIG. 3. Analysis of κ RNA transcripts in the transfectant κ-2. Poly(A)-containing RNA was isolated from cells, size-fractionated by agarose gel electrophoresis, transferred to nitrocellulose paper, and hybridized with ^{32}P-labeled DNA from a plasmid containing the sequence from J_κ through C_κ. Lanes a and b, RNA from the transfectant κ-2 grown in the absence (lane a) or presence (lane b) of LPS. Lane c, RNA from the κ-producing A-MuLV-transformed cell line 18-48.

ent line 81A-2 (not shown), but RNA from the κ-2 line contained hybridizing species of approximately 1.2, 1.9, and 2.6 kilobases (kb) (Fig. 3, lane a). The smaller RNA comigrated with authentic κ mRNA from the A-MuLV-transformed cell line 18-48 (Fig. 3, lane c). [The smaller 0.8-kb RNA in 18-48 is an aberrantly small κ transcript (5).] In other experiments, both the 1.9-kb and the 2.6-kb RNAs were found to hybridize strongly to a probe specific for the intervening sequence between J_κ and C_κ and hybridize weakly to a pBR322 DNA probe. Hence, these higher molecular weight species are some type of aberrant RNA. The 1.2-kb species, however, appears to be an authentic κ mRNA transcript in that it hybridizes only to the probe containing κ coding sequences and not to the intervening sequence probe or pBR322.

Because many A-MuLV-transformed lymphoid cell lines increase Ig production when LPS is added to the medium (18, 31), we investigated the effects of LPS on κ chain synthesis in the transfected line. The parent line 81A-2 increases synthesis of γ2b heavy chain protein and mRNA upon induction by LPS (22) (Fig. 2, lane c). When LPS was added to the κ-2 cells, κ light chain synthesis increased approximately 5-fold, to a level approximately 1/15th that of the myeloma MPC11 (Fig. 2, lane e). To determine if the LPS-induced increase in κ chain synthesis was due to an increased content of specific mRNA, the mRNA fraction was prepared from LPS-treated 81A-2 parent cells and transfected κ-2 cells. Again, no κ mRNA species was

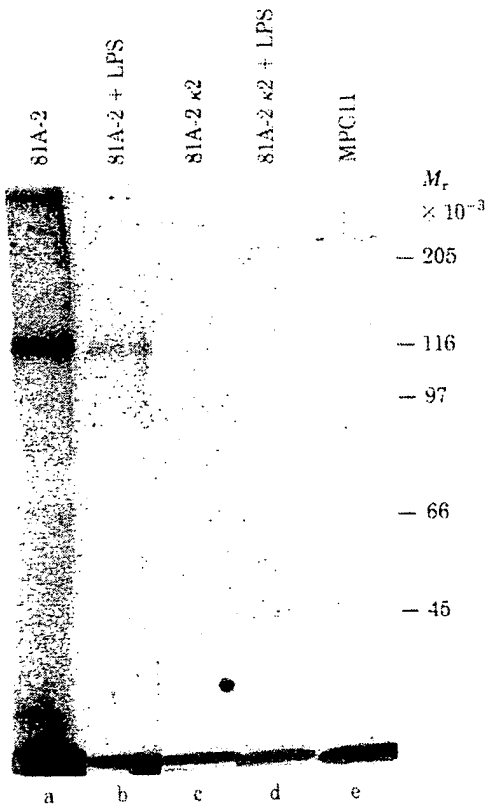

FIG. 4. Polyacrylamide gel electrophoresis of nonreduced Ig synthesized by the κ-2 transfectant. Cytoplasmic extracts were prepared as for Fig. 2. Lanes a and b, extracts from the parent 81A-2 cells grown without (lane a) and with (lane b) LPS and immunoprecipitated with anti-κ antiserum; lanes c and d, extract from κ-2 transfectants grown without (lane c) and with (lane d) LPS and immunoprecipitated with anti-κ antiserum; lane e, myeloma MPC11 extract immunoprecipitated with anti-κ antiserum.

detected in the parental cells, but the κ-2 cells contained increased levels of the 1.2- and 1.9-kb species (Fig. 3, lane b). Interestingly, the level of the 1.9-kb RNA species increased even more than that of the presumably authentic 1.2-kb RNA species.

Because the transfected cells were producing both γ2b heavy chains and κ light chains, it was possible that the cells could assemble the heavy and light chains into IgG. To examine this question, samples of [^{35}S]methionine-labeled protein extracts were immunoprecipitated with anti-κ antiserum and the nonreduced samples were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. The parental 81A-2 cells produced a protein of approximately the correct size for γ2b heavy chain dimers (Fig. 4, lanes a and b; the darker appearance of lane a is due to more labeled extract present). The κ-2 cells produced a protein that migrated slightly faster than the IgG2b produced by the myeloma MPC11 (Fig. 4, lanes d and e) but slower than the bulk of the rabbit IgG antiserum visualized by staining (not shown). In other experiments (not shown) no free κ chain was found in the κ-2 cells, although a significant amount was present in MPC11 cells. Essentially all of the κ chain produced in the κ-2 cells appears to be assembled into IgG2b.

DISCUSSION

The major result of these studies is the demonstration that a functional κ gene can be introduced into a lymphoid cell line in which it will be continuously expressed. This opens the possibility of examining control and rearrangement mechanisms in

lymphoid cells by using inserted genetic elements.

The κ gene introduced into 81A-2 cells apparently functions normally in spite of being in a very unusual context. The gene was in an SV40/pBR322 vector that then integrated into a presumably random site in the cell genome, a site unlikely to be related to the normal location of the κ gene in chromosome 6. In spite of its unusual context, the introduced gene was expressed at about the same level as the resident γ2b heavy chain gene. The κ gene was apparently using its own promoter because in the construction no promoter was provided that faced in the correct direction. It is possible that the SV40 DNA sequences present might have provided some enhancing function for κ expression (32).

The introduced κ gene not only was expressed at a basal level but also was inducible by LPS. The mechanism and function of this induction system are far from clear, but the ability of the introduced κ gene to respond indicates that sufficient κ-related DNA sequences to provide for LPS inducibility were included in the construct. The construct contained, in addition to the V_κ, J_κ, and C_κ coding segments, the intervening sequence between the coding regions and about 1–1.5 kb of DNA both 5′ of $V_\kappa J_\kappa$ and 3′ of C_κ. Any of this extra DNA could be involved in promoter and control functions, but the results make it unlikely that any sequences important for κ expression exist more than 1.5 kb to either side of the coding region.

LPS control of heavy chain expression in 81A-2 cells is allele specific and correlates with a deletion in the intervening sequence between $V_H DJ_H$ and C_μ (22, 33). The productively rearranged heavy chain allele is inducible by LPS and contains this deletion, whereas the other allele, containing a nonproductive rearrangement, lacks the deletion and is not inducible by LPS. Therefore, LPS inducibility of heavy chain seems to be determined at the DNA level. Whether the introduced κ gene is responding directly to LPS or to the product of the heavy chain allele is an open question. The possibility that transcription of the light chain gene is controlled by a product of the heavy chain locus is an interesting possibility and needs further investigation.

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